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Polyphenolic content and antioxidant activity of *Hyssopus officinalis* L. from the Republic of Moldova

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Abstract

Background: *Hyssopus officinalis* L. (hyssop), a species native to the Caspian Sea region, has been cultivated in the Republic of Moldova as aromatic plant and has been used in folk medicine as antitussive, expectorant, carminative, digestive and sedative remedy.
Material and methods: The aerial parts of *H. officinalis* L., with pink, white and blue flowers, were collected from the collection of the Scientific Practical Center in the Field of Medicinal Plants during flowering phase. The extracts were obtained with 70% ethanol by maceration with stirring. The concetration of the extracts was done with the rotative evaporator Laborota 4011. Identification of phenolic compounds in dried extracts from hyssop herb was carried out by thin-layer chromatography. The total content of hydrocyanamic acids was measured in plant products and dried ethanolic extracts, by spectrophotometric method, with Arnow's reagent. Quantitative analysis of total phenolic content was carried out by UV-spectrophotometry analysis, using a Metertech UV/VIS SP 8001 spectrophotometer.
Results: The chlorogenic (Rf 0.47) and caffeic (Rf 0.93) acids were identified in the three genotypes of *Hyssop herba,* with pink, white and blue flowers. The total of hydroxycinnamic acids, expressed in caffeic acid, for both, aerial parts and dry extract, was the highest in *H. officinalis* L. with white flowers (1.484 mg/g; 3.014 mg/g respectively), followed by *H. officinalis* L. with pink flowers (1.190 mg/g; 2.915 mg/g) and *H. officinalis* L. with blue flowers (1.015 mg/g; 2.851 mg/g).
The highest polyphenol content, expressed in gallic acid equivalent (GAE), was found in the dry extract of *H. officinalis* L. with blue flowers (39.056 mgGAE/g dry extract).
Conclusions: This study showed that the extract of the *Hyssopus officinalis* L. containing phenolic compounds, can be used as a natural antioxidant in pharmaceutical and cosmetic industries.
Key words: *Hyssopus officinalis* L, phenolic compounds, antioxidant activity.

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Introduction

Phenolic compounds are members of the largest group of plant secondary metabolites and have the main function to protect the plants against ultraviolet radiation or invasion by pathogens [1]. They can be divided into four distinct classes based on the number of phenol rings and structural fragments connecting them, namely phenolic acids, flavonoids, stilbenes and lignans [2]. The first class generally involves the phenolic compounds possessing a carboxylic acid as the main functional group [3], thus being named as phenolic acids, which are further split into two groups, namely hydroxybenzoic and hydroxycinnamic acids [4].

Hydroxycinnamic acid derivatives comprise a large group of simple phenolic acids, are abundant in fruits, vegetables and cereals and seeds of fruits. Ferulic acid, caffeic acid, p-coumaric acid, chlorgenic acid, sinapic acid, curcumin and rosmarinic acid belong to this important phenolic acid group [3].

Hyssopus officinalis L. (hyssop) belonging to the family Lamiaceae, is a perennial herbaceous plant from Southern Europe and some temperate regions of Asia. Hyssop, which is one of the most important pharmaceutical herbs, is extensively cultivated in central and South European countries, such as Spain, France and Italy [4]. Hyssop has thick ramified roots and several 20-60 cm high wooden stems. This plant has small, paired, pointed and very fragrant leaves and purplish darkblue, white and occasionally red flowers [5-7].

Hyssop has been exploited for many uses. It is well known for its aromatic scent, and as an ornamental and bee attracting plant. The aerial parts are used in the food industry as a condiment and spice or as a minty flavor. In traditional medicine, the plant has long been used as

a carminative, tonic, antiseptic, expectorant and cough reliever [8].

The aerial shoots of this herbaceous perennial are useful for the treatment of respiratory diseases, including asthma, bronchitis and cough, as they contain chemicals, such as terpenes, flavonoids, volatile oils, tannins and resins [5]. In *H. officinalis* L., from flavonoids group, apigenin, quercetin, diosmin, luteolin and their glucosides, and from other phenolic compounds – chlorogenic, protocatechuic, ferulic, syringic, p-hydroxybenzoic and caffeic acids are present. Reports on the essential oils extracted from aerial parts of *H. officinalis* L. revealed several principal components, including terpenoids pinocamphone, isopinocamphone and β-pinene [9].

The extracts and the essential oil isolated from hyssop showed moderate antioxidant and antimicrobial activity together with antifungal and insecticidal antiviral properties, *in vitro*. The ethanol extract of hyssop has been recently reported to present protective properties for the gastric tract based on the adhesive stomach mucosal compounds, and also significant anticoagulant and antioxidant properties through reducing the production of free radicals [5]. Animal model studies indicated myorelaxant, antiplatelet, and α-glucosidase inhibitory activities for this plant. The essential oil is mainly used for flavouring and food preservation and for phytotherapeutic uses [10]. Research suggests that hyssop essence has special antimicrobial effects on bacteria, such as *Streptococcus pyogenes*, *Staphylococcus aureus, Escherichia coli* and *Candida albicans* [5].

The purpose of this study was to investigate the content of the secondary metabolites accumulated in the aerial parts of hyssop cultivated in the Republic of Moldova, comparison of 3 genotypes with distinctive morphological characters highlighted by the colour of flowers (blue, pink and white) and evaluation of polyphenolic content and antioxidant capacity of the extractive products of the plant.

Material and methods

As plant material, the aerial parts of 3 genotypes of *Hyssopus officinalis* L. – hyssop with blue flowers, hyssop with pink and white flowers, collected from the Scientific Practical Center in the Field of Medicinal Plants of *Nicolae Testemitanu* State University of Medicine and Pharmacy, were used. The product was sampled during July 2022.

Obtaining the dry extract by the fractional maceration method

The method of fractional maceration with stirring was used for the extraction. 10 g of chopped plant product (*Hyssopi officinalis herba*) were treated with 5 portions of 200 ml ethanol 70% each, in a ratio of 1:10. All extraction phases lasted 60 min, with the separation of the extractive liquid from the vegetable residue. Fractions of extractive solutions stayed in the refrigerator for 5-6 hours at +50°C, then they were filtered through Whatman filter paper No 2, using the Buchner funnel. The Laborota 4011 digital rotary evaporator was used to concentrate the extractive solutions. The alcohol was evaporated at 40°C. The stirring was carried out with a magnetic stirrer at room temperature [11, 12].

Qualitative analysis by Thin Layer *chromatography* (TLC)

The qualitative evaluation of the alcoholic plant extractions was carried out on a silica TLC plate. The time of extraction was 60 min. Test solution: 0.05 g of dry extract was dissolved in 70% ethanol in a 25 ml volumetric flask. Reference solution: 0.1% solutions of rutin, hyperoside, isoquercetin, quercetol, apigenin, luteolin, gallic acid, chlorogenic acid, caffeic acid. Stationary phase: silica gel plates. Mobile phase: ethyl acetate: formic acid: water (6:9:90). Migration: 12.4 (extraction duration 1 hour) – 12.5 (extraction duration 15 min) cm. Plate drying: 100-105°C for 10 min. Detection: The plate was sprayed with a 3% solution of $AlCl_3$. After 30 min the plate was examined under UV light at a wavelength of 366 nm [12].

Determination of total hydroxycinnamic acids

The preparation of the stock solution from aerial plant products was performed with 80 ml of 50% ethyl alcohol mixed with 0.2 g of crushed plant product, boiled on a water bath under reflux condenser for 30 minutes, then cooled and filtered into a volumetric flask with 100 ml capacity. The entire volume was adjusted to the mark with 50% ethanol. Stock solution of dry extracts: 0.01 g of dry extract was dissolved with 80% ethanol, brought up to level in a 10 ml volumetric flask with the same solvent. The test solution: 1 ml of stock solution, 2 ml of 0.5 M hydrochloric acid, 2 ml of Arnow's reagent (prepared by dissolving 10 g of sodium nitrite and 10 g of sodium molybdate in 100 ml of water), 2 ml of 8.5% sodium hydroxide solution, adjusted up to the mark of 10 ml with purified water. The whole mixture was stirred. Blank solution: 1 ml of the stock solution adjusted up to 10 ml with distilled water. The absorbance of the test solution was immediately measured at 518 nm [13].

Determination of total polyphenol content

The quantitative analysis of the polyphenols, in the plant products of hyssop and in the dry extracts, was carried out by the spectrophotometric method with the Folin-Ciocalteu reagent, using gallic acid as standard. The samples from the dried hyssop extracts were prepared as 1 mg/ml with 70% ethyl alcohol. From each solution obtained, an aliquot of 1 ml, 5 ml of Folin-Ciocalteu reagent (1:10) was added, and stirred for one minute. After that 4 ml of 4% sodium carbonate solution were added. After stirring the mixture was left for 2 hours at room temperature in a dark place. The absorbance on Metertech UV/VIS SP8001 spectrophotometer at a wavelength of 765 nm was measured. Total polyphenolic content was expressed in mg gallic acid equivalent/g absolute dry mass of hyssop extract. The total polyphenol in the analyzed samples was

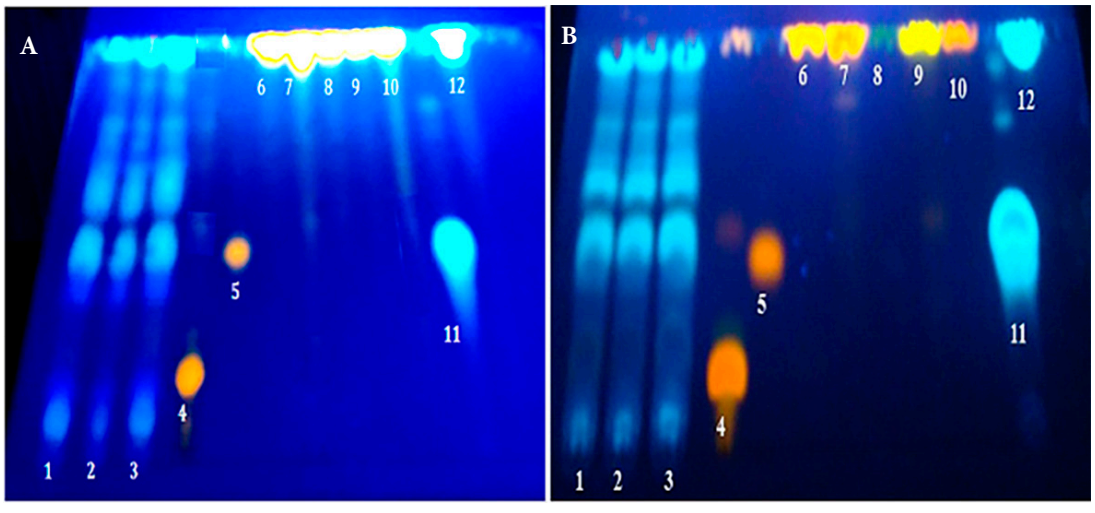

Fig. 1. Chromatogram of plant products (A) and dried extracts (B) of *Hyssopus officinalis* L. under UV light 366 nm

Samples from aerial parts with: 1 – white flowers, 2 – pink flowers, 3 – blue flowers. Standard substances: 4 – rutin, 5 – hyperoxide, 6 – isoquercetol, 7 – quercetol, 8 – apigenin, 9 – luteolin, 10 – myricetol, 11 – chlorogenic acid, 12 – caffeic acid

calculated using the calibration curve established under the same conditions as the analyzed solutions. All measurements were performed 3 times [14].

Determination of the antioxidant activity by the DPPH method

Stock solution: in a 100 ml volumetric flask, 20 mg of 1,1-diphenyl-2-picrylhydrazyl (DPPH) were added and adjusted up to the mark with 96% ethyl alcohol (concentration 200 mg/l). The given solution was kept at +4°C, in aluminum foil, for no more than a week. 5 ml of stock solution were withdrawn to a 50 ml volumetric flask and adjusted up to the mark with 96% ethanol (concentration 1 mg/ml). From this solution, the dilutions for the calibration curve (200, 100, 75, 50, 25, 10, 5 μg/ml) were prepared. The test solution: 1.5 ml of ethanolic solution was added to 3 ml of DPPH solution (20 mg/l). Absorbance was measured at a wavelength of 517 nm, 96% ethyl alcohol was used as blank solution [15-17].

All chemicals used were of analytical grade. Gallic acid, rutin, hyperoside, isoquercetin, quercetol, apigenin, luteolin, chlorogenic acid, caffeic acid, sodium hydroxide, sodium nitrite, Trolox, DPPH and Folin-Ciocalteu reagent all were obtained from Sigma Aldrich Corporation.

The experiments were carried out in triplicates and statistical analysis was performed by Excel 2020 using $p< 0.05$ significance level.

Results and discussion

Identification of flavonoids by TLC was performed to identify flavonoids in plant products and dry extracts. The extracts were dissolved in 70% ethanol. The analyzed samples and the controls were applied to the chromatographic plate (20x20 cm). After the development, the yellow color predominated, but the shades were different. The spots were analyzed with an ultraviolet lamp (fig. 1). To identify the substances, the Rf value and fluorescence of the spots obtained with the sample solution and the Rf value and fluorescence of the spots obtained with the reference solution were compared.

Thus, after analyzing the chromatogram in UV light at λ = 366 nm it was observed that in all the three genotypes of *Hyssopus officinalis* L. (white, pink and blue flowers), the blue spots corresponding to numbers 8 and 9 could be clearly observed, which denotes the presence of caffeic and chlorogenic acids. Also, in addition to the identified substances, a number of substances were separated on the plate, which demonstrates the diversity of compounds from the aerial parts of hyssop. The qualitative analysis by TLC demonstrated the presence, in plant products, along with the dry extracts, obtained from the 3 genotypes of *H. officinalis* L., of chemical compounds from flavonoid group: rutin (Rf = 0.21), caffeic acid (Rf = 0.93) and chlorogenic acid (Rf = 0.47), the chromatograms of the genotypes being practically identical.

Quantitative determination of total hydroxycinnamic acids

The European Pharmacopoeia method, for determining the total hydroxycinnamic acids (THA), describes the determination of the THA in the aerial parts of the plant and in its dry extract, using the Arnow's reagent (10% aqueous solution of sodium nitrite and sodium molybdate, 2 ml). Analysis of each sample was performed in triplicate. Following the experience, it was observed that the leader among the genotypes with the highest content of THA expressed in caffeic acid was the *H. officinalis* L. with white flowers (1.484±0.620 mg/g) as shown in fig. 2; the lowest content of THA was determined in *H. officinalis* L. with blue flowers (1.015±0.024 mg/g).

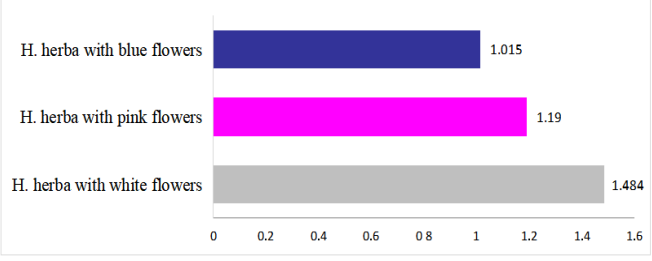

Fig. 2. The total content of hydroxycinnamic acids in plant products from the aerial parts of *Hyssopus officinalis* L. (mg/g)

The evaluation of THA in the hyssop dry extracts, revealed that the richest content was present in the dried extract of *H. officinalis* L. with white flowers (3.014±0.114 mg/g) followed by *H. officinalis* L. with pink flowers (2.915±0.126 mg/g) and *H. officinalis* L. with blue flowers (2.851±0.103 mg/g).

Quantitative determination of total polyphenols in plant products and dry extracts from the aerial parts of *Hyssopus officinalis* L. was identified by the spectrophotometric method in recalculation to gallic acid. The absorbance was read on a UV-VIS spectrophotometer over 90 min at a wavelength of 765 nm. The calibration curve was prepared in the following concentrations: 0.01; 0.02; 0.03; 0.06; 0.08 mg/ml (fig. 3) in methanol from standard 1 solution of gallic acid.

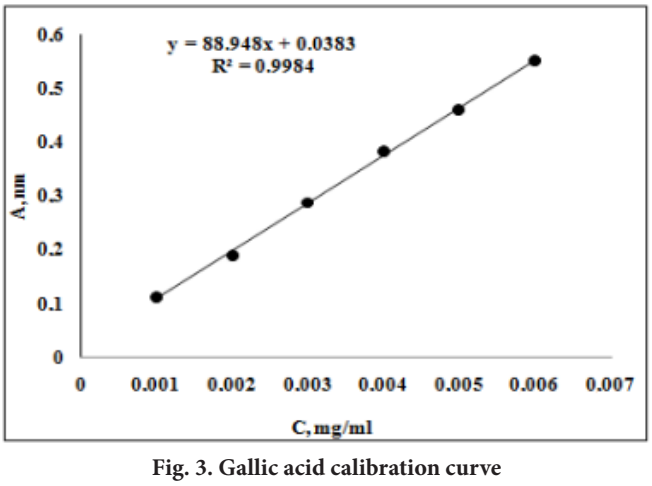

Fig. 3. Gallic acid calibration curve

The total polyphenol content (TPC) was ca culated according to the following formula: x=(y+0.0383)×250) /88.948 in gallic acid equivalent mg/g dry mass. The highest amount of phenolic componds was determined in aerial parts of hyssop with blue flowers (12.256±0.120 mg-GAE/g) and the lowest content in aerial parts of hyssop with white flowers (8.012±0.059 mgGAE/g), as shown in fig. 4.

The spectrophotometric dosage of TPC in dry extracts, demonstrated that the highest content was found in *H. officinalis* L. with blue flowers (39.056±0.894 mg/g dry extract), followed by the dry extract of *H. officinalis* L. with white flowers (36.111±0.314 mg/g) and the dry extract of *H. officinalis* L. with pink flowers (33.078±0.620 mg/g). The obtained results demonstrated the advantage of concentration of liquid extractive products and solvent evaporation, thus increasing the content of phenolic compounds.

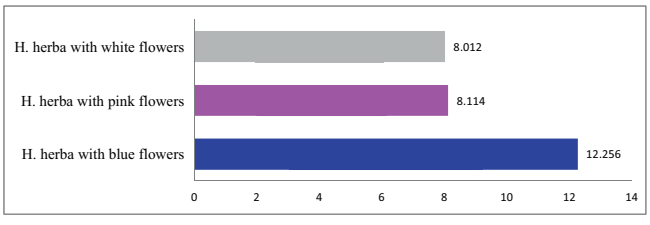

Fig. 4 The content of total polyphenols in plant products from the aerial parts of *Hyssopus officinalis* L. (mgGAE/g)

There are only few reports on the polyphenolic content of hyssop and no comparisons of different accessions of the species. Németh-Zámbori E. et al. reported significant difference in the total phenolic content among genotypes, that ranged from 443.64 mg/g DW ('Erfurter Ysop') and 329.32 mg/g DW ('Hyzop lekarsky') calculated as gallic acid. The effect of the year was not significant, although, detected a significant variety in year interaction for the species cultivated in Hungary [18].

Determination of the antioxidant activity by the DPPH method

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical is a stable radical with a maximum absorption at 517 nm that can readily undergo reduction by an antioxidant. Because of the ease and convenience of this reaction it now has widespread use in the free radical-scavenging activity assessment [19]. Determination of the antioxidant action using the DPPH test (tab. 1), revealed that all three studied hyssop genotypes, *H. officinalis* L. with blue flowers (IC_{50}=38.091 mg/ml), *H. officinalis* L. with pink flowers (IC_{50}=34.172 mg/ml) as well as *H. officinalis* L. with white flowers (IC_{50}=34.774 mg/ml) possess high antioxidant properties, compared to the standard substance Trolox (IC_{50}=3.15 mg/ml).

Table 1. Total phenolic content and antioxidant activity of dry extracts from aerial parts of different *Hyssopus officinalis* L. genotypes

Hyssop samples	TPC mg GAE/g	THA mg CA/g	IC_{50}, mg/ml M±SD
Dry extract of *H. herba* with white flowers	36.111±0.314	3.014±0.114	34.774±1.954
Dry extract of *H. herba* with pink flowers	33.078±0.620	2.915±0.126	34.172±0.682
Dry extract of *H. herba* with blue flowers	39.056±0.894	2.851±0.103	38.091±0.288
Trolox			3.15±0.147

Each value is the mean ± SD of three independent measurements

Fathiazad F. et al. demonstrated the total phenolic content in the n-butanol and ethylacetate extracts of the aerial parts of *H. officinalis* L. from Iran was found to be

246 mg gallic acid equivalent GAE/g and 51 mg GAE/g [20]. Comparing the polyphenolic content, the methanolic extract obtained from Iranian *H. officinalis* L. var. *angustifolius* was richer than the ethanolic extract obtained from Romanian hyssop (90 mg/g and 77.72 mg/g, respectively) [10]. The antioxidant activity of ethanolic extract of *H. officinalis* L. (IC_{50}=125.44±4.70 μg/ml), native to Romania, was evaluated by DPPH radical scavenging, with Trolox as a positive control (IC_{50} = 11.20±0.20 μg/ml) [10].

The contents of total polyphenols in six samples of *H. officinalis* L. were collected from five localities on the territory of Montenegro, ranged between 64.1 and 112.0 mg GAE/g. The content of chlorogenic acid was in the range between 23.35 and 33.46 mg/g, whereas rosmarinic acid was present in lower amounts (3.53–17.98 mg/g). The sample richest in chlorogenic and rosmarinic acids was also the richest in total polyphenols. The methanol extracts expressed moderate to weak antioxidant activity (DPPH IC_{50} = 56.04-199.89 μg/ml), compared to the rutin methanolic solution [21].

Ethanolic extracts of *H. officinalis* L. var. angustifolius stems, leaves, and flowers showed good antioxidant activity. Inhibitory concentration (IC_{50}) for DPPH scavenging was found to be 148.8±4.31 μg/ml for flowers, 208.2±6.45 μg/ml for leaves and 79.9±2.63 μg/ml for stems [22].

Conclusions

The experimental findings show that dry extracts of hyssop cultivated in the Republic of Moldova, contain considerable amounts of hydroxycinnamic acids and polyphenols due to which, the free radical scavenging power, determined with DPPH method is comparable with Trolox standard. The findings of this study specify the importance of *Hyssopus officinalis* L. as a readily available source of antioxidants in order to prevent the occurrence of neurosis and hyper-excitability, liver dysfunction, inflammatory diseases for which free radicals are considered important contributing factors.

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Authors' contributions
AB designed the study, performed the laboratory work and drafted the first manuscript; CC interpreted the data, revised the manuscript; IP introduced into culture the species *Hyssopus officinalis* L., NC conducted the laboratory work; MC-T revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

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Ethics approval and consent to participate
No approval was required for this study.

Conflict of interests
No competing interests were disclosed.